'IN VIVO' ROLE OF 'PSEUDOMONAS AERUGINOSA' TOXINS AND HOST RESPONSE

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"*In Vivo* Role of *Pseudomonas aeruginosa* Toxins and Host Response"

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13 ABSTRACT

In vitro and in vivo studies were performed on both endotoxin and collagenase obtained from Pseudomonas aeruginosa. The collagenase was extensively purified by both chemical and chromatographic technics and was subsequently assayed for potential toxic activity. The collagenase yielded a 72 hr LD50 value in mice of 148 enzyme units (I.P.), 289 units (I.V.) and 55 units (intranasally). The subcutaneous route was not lethal although black, necrotic, ulcerating lesions were produced. Intranasal instillation of collagenase resulted in confluent pulmonary hemorrhage, while I.P. injections resulted in severe abdominal hemorrhage with foci on the peritoneal membrane and intestinal serosa. Intravenous injections elicited abdominal hemorrhage and petechial hemorrhage with focal necrosis of the lungs.

The endotoxin from Pseudomonas aeruginosa C_9 was obtained by five standard methods of extraction for comparative electron microscopic studies. Observation of the five preparations demonstrated the presence of two major types of structures. The first of these was seen in endotoxins prepared by trichloroacetic acid, ethyl ether, hot water, and ethylenediaminetetraacetate-lysozyme extraction, and consisted of discrete spherules containing small spherules within and having a homogeneous staining center and rod-like border. The other morphologic type was seen only in preparations obtained by the aqueous phenol technique and consisted of pleomorphic staining material and rodlets. Preparations isolated by trichloroacetic acid extraction could be morphologically converted to the same appearance as aqueous phenol preparations by phenol extraction. Loss in structural integrity was encountered upon exposure to polymyxin B, colistin sulfate and carbenicillin, but not with other antibiotics tested

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SUMMARY

The endotoxin from *Pseudomonas aeruginosa* C_9 was obtained by five standard methods of extraction for comparative electron microscopic studies. Observation of the five preparations demonstrated the presence of two major types of structures. The first of these was seen in endotoxins prepared by trichloroacetic acid, ethyl ether, hot water, and ethylenediaminetetraacetate-lysozyme extraction, and consisted of discrete spherules containing smaller spherules within and having a homogeneous staining center and rod-like border. The other morphologic type was seen only in preparations obtained by the aqueous phenol technique and consisted of pleomorphic staining material and rodlets. Preparations isolated by trichloroacetic acid extraction could be morphologically converted to the same appearance as aqueous phenol preparations by phenol extraction. Loss in structural integrity was encountered upon exposure to polymyxin B, colistin sulfate and carbenicillin, but not with other antibiotics tested.

An extracellular protease from *Pseudomonas aeruginosa* having collagenase activity was assayed *in vivo*. The lethality of the enzyme for white female mice was determined by use of intravenous, intraperitoneal, intranasal, and subcutaneous routes, respectively. The collagenase exhibited the following 72-hr LD_{50} values: Intranasally, 55 collagenase units; intraperitoneally, 148 collagenase units; and intravenously, 288 collagenase units. In the concentrations tested no lethality was obtained when the subcutaneous route was

employed. Gross and microscopic studies revealed that the collagenase was capable of eliciting a variety of tissue responses in mice depending upon its route of administration. Intranasal instillation resulted in confluent pulmonary hemorrhage, while intraperitoneal injections resulted in severe abdominal hemorrhage with foci on the peritoneal membrane and intestinal serosa. Intravenous injections elicited abdominal hemorrhage and petechial hemorrhage with focal necrosis of the lungs, while subcutaneous injections resulted in black, necrotic, ulcerating lesions.

RESULTS

Electron Microscope Characterization of Endotoxin. Studies to determine the ultrastructural character of the endotoxin extracted by the five procedures was undertaken. Two morphologically distinct groups of structures were observed. The first of these was seen only in material obtained by aqueous phenol extraction. This material contained a pleomorphic globular material as well as rodlets. The rodlets were of fairly uniform diameter (6.6 nm) but varied considerably in length. The material exhibited a 72 hr mouse LD_{50} of 450 µg when administered intravenously as previously described (Dyke and Berk, 1972). The second structural type observed in the study was seen with the remaining four preparations. This form consisted of small, irregular sphere-like particles which contained additional spherules within and appeared to be more homogeneous in appearance. Most of the particles appeared to have a diameter of approximately 100 nm. The outer edge of the sphere-like particles appeared to be structurally similar to the rodlets seen in the aqueous phenol extracts. The term "associated form" was given to the spherules seen in endotoxins prepared by TCA, ethyl ether, EDTA-lysozyme, and hot water extraction. The appearance of the aqueous phenol prepared endotoxin was suggestive of an altered or disrupted phase of the "associated" type and so was termed the "dissociated" form.

Structural Alteration Studies. Since there was a structural identity between preparations yielding the "associated" type of structure, the TCA product was used as a representative model for

this morphologic form. It exhibited a 72 hr mouse LD_{50} of 589 µg when administered intravenously (Dyke and Berk, 1972). The first set of experiments was designed to determine the stability of the "associated" and "dissociated" structural forms to various hydrogen ion concentrations. No ultrastructural changes could be detected in a pH range from 5.7 to 8.0 using 0.1 M phosphate. However, when the pH was lowered below 4.0 (0.1 M glycine-HCl buffer) or raised to 9.2 or above, (0.1 M carbonate-bicarbonate buffer), both preparations formed insoluble aggregates which could not be resolubilized upon dilution. Because of the nature of the aggregates visualization of their structure with the electron microscope was impossible. Attempts were then undertaken to convert the "associated" structure obtained with the majority of the extraction products to the "dissociated" structure as seen in aqueous phenol preparations. When the "associated" form was treated with hot aqueous phenol (Westphal et al, 1952) the spherules appeared to become disrupted and the structure obtained was similar to that originally obtained by direct phenol extraction. Consequently, the spherules were no longer found intact, but instead small rodlets and pleomorphic staining material was now the major structural feature. Intermediate forms could be frequently found in these treated preparations which may have resulted from a partial opening of the outer ring of the spherule with internal constituents seen to be flowing out. The internal substance appeared to have a consistency similar to that of the pleomorphic material observed in the "dissociated" form.

Treatment of "associated" and "dissociated" forms by various enzymes, chemicals and antibiotics was also studied. The enzymes used included the following: subtilopeptidase, trypsin, lipase, phospholipase C, alkaline phosphatase, hyaluronidase, and muramidase. Of the enzymes tested only the lipase was found to produce visible alterations. Of particular interest was the fact that although subtilopeptidase did not alter the structure of either endotoxin form, approximately 50% of the "associated" form appeared to stain abnormally so that an electron translucent area within could be seen. The significance of this occurrence is not yet understood, especially since no effect was noted with the "dissociated" form. The action of wheat germ lipase was somewhat selective in that no significant visible effects were noted on the "associated" form while some structure altering effects could be seen with the "dissociated" form. The pleomorphic constituents of the "dissociated" form was further dispersed and no globular elements could be found. In addition, many of the rodlets appeared to be fragmented and were present as shorter segments. The structure-altering properties of lipase were also time-dependent. Changes could not be detected immediately but required an incubation of at least 2 h. However, increased changes were not evident upon extended incubation. In addition, no structural changes were noted with trypsin, phospholipase C, alkaline phosphatase, and hyaluronidase, although 0.4% sodium dodecyl sulfate and muramidase immediately caused precipitation at either 4 or 37°C. Incubation of the endotoxin preparations with equal proportions of human plasma or guinea pig complement also had no visible effect on their structure.

The antibiotic colistin sulfate (125 µg/ml) produced organizational changes in both the "associated" and "dissociated" forms. However, the degree of change was less dramatic upon the "associated" spherules. Colistin sulfate (125 µg/ml) caused the disruption of many spherules; however, the effect was only partial since intact spherules could still be detected even after incubation for 18 h. The outer ring of disrupted particles were broken into small units and appeared somewhat like the rodlets earlier described. The main effect of colistin sulfate on the "dissociated" form appeared to be the formation of ringlets resulting in a chain-like appearance. The amorphous material was no longer apparent and may have been converted to packets of ringlets. Also, there was a great increase in rodlets which appear as long strands of ringlets unwinding from the packets. There was also suggestive evidence that the individual ringlets separated and were found free. Polymyxin B (12.5 µg/ml) appeared to exert an identical effect on both "associated" and "dissociated" forms. In both preparations there is a conspicuous absence of either free rodlets or the rod-like border around intact spherules and there appears to be remnants of this component in various stages of disintegration. The appearance of fused ringlets is identical to that obtained with *E. coli* endotoxin treated with polymyxin (Koike and Iida, 1971). The internal constituents of the "associated" form seems to be little affected by the action of the drug. Results comparable to those seen with polymyxin B were also obtained with carbenicillin (125 µg/ml). The morphologic appearance seen in both "associated" and "dissociated" types of endotoxin following treatment with carbenicillin indicated that intact rodlets could not be observed in either preparation nor was

there a detectable rod-like border remaining on the "associated" form. The remaining antibiotics, streptomycin, mitomycin C, actinomycin D, and garamycin had no visible altering effect on either form of extracted endotoxin.

COLLAGENASE STUDIES

Initial studies were designed to determine the LD_{50} values for purified collagenase from *P. aeruginosa* when administered to mice by the intranasal, intraperitoneal, intravenous, and subcutaneous routes, respectively. Concomitantly, using the same levels of enzyme protein, comparative studies with heat-inactivated enzyme were also performed in an attempt to determine whether there was a relationship between lethality and enzymatic activity. A summary of the LD_{50} values for the active enzyme administered by the four different routes can be seen in Table 1 and indicate that the same pattern of lethality was obtained as preciously described with the *P. aeruginosa* elastase. Consequently, the animals were most susceptible to the collagenase by the intranasal route, followed by the intraperitoneal and intravenous route, respectively. No lethality was obtained by use of the subcutaneous route within the concentration range tested. For the gross and histopathology studies animals were administered LD_{50} values as well as above and below them for each route and were autopsied within minutes of death. When animals survived longer than 72 hr, they were sacrificed by cervical detachment along with control animals and then autopsied.

Intranasal Route. The LD_{50} value for the intranasal route was found to be 55 enzyme units (61.8 µg protein). A dose response was apparent since the severity of the toxic response increased as an increase in enzyme units was administered. When the 72 hr LD_{50} dose of 55 collagenase units was employed the animals exhibited severe pulmonary distress prior to death as illustrated by belabored

breathing and gasping for air in a series of violent jumping spasms. Whereas animals receiving doses above the LD_{50} such as 90 units died within a few minutes with similar respiratory distress. The gross pathology observations indicated a severe confluent pulmonary hemorrhage encompassing large areas extensively damaged with almost complete loss in structural integrity noted 5 min after administration of 90 enzyme units (100 µg protein), whereas the LD_{50} dose of 55 enzyme units (61.8 µg protein) gave essentially the same gross pathology response within 24 to 72 hr. With the latter dose large amounts of blood were found in the thoracic cavity and bleeding from the nose and mouth was also extensive. Histologically, extensive pulmonary hemorrhage and necrosis was observed along with capillary hyperemia, and the bronchioles were flooded with blood. In addition, pyknotic nuclei were seen throughout the tissues. No other organs were significantly affected, although slight venous congestion of the liver was occasionally observed within 6 to 72 hr. Doses below the LD_{50} value such as 9 to 45 units resulted in less severe hemorrhage in regard to total area involved; however, when death occurred, it involved confluent pulmonary hemorrhage.

Intraperitoneal Route. Animals receiving intraperitoneal injections of the LD_{50} dose of 148 enzyme units (164.8 µg protein) experienced impairment of caudal mobility which in terminal animals progressed to complete loss of this activity. Terminal animals also exhibited severe hindleg and sacral spasmotic muscle contractions. Gross pathology studies indicated the presence of massive abdominal hemorrhage with hemorrhage in the intestinal serosa within 24 hr to

72 hr. Doses of 154 to 248 enzyme units (171 to 275 µg protein) yielded the same results within 1 hr. Doses below the LD_{50} also produced extensive abdominal hemorrhage, but many of the animals survived the challenge. Histologically, administration of the LD_{50} dose resulted in hemorrhage and destruction throughout the duodenal serosa, muscularis, submucosa and mucosa. In addition, hemorrhage of the pancreas, spleen and liver was also noted. However, no pulmonary hemorrhage or tissue damage was ever noted. Trichrome stained sections of the intestinal tract suggested that in the areas of focal hemorrhage and necrosis of the intestinal wall, collagen was either removed or destroyed.

Intravenous Route. Animals receiving the LD_{50} dose of 288 enzyme units (318 µg protein) showed an initial decrease in activity with ruffled fur and encrusted eyes. Animals surviving the 72 hr holding period made a marked recovery and thereafter showed none of these symptoms. With doses above the LD_{50} value, such as 350 units, animals showed marked pulmonary distress and death within a few minutes with bleeding from the nose and mouth. The gross pathology obtained with 288 enzyme units consisted of petechial hemorrhage with focal necrosis in the lungs plus abdominal hemorrhage within 24 to 72 hr. In addition, the lumen was filled with half-digested blood. With doses above the LD_{50} value (300-325 units), severe hemorrhage and necrosis of the lungs could be obtained within 3 hr, while a dose of 250 units yielded milder pulmonary damage. Histologically, with the LD_{50} dose, striking lung hemorrhage, necrosis, and destruction of the integrity of the pulmonary parenchyma with capillary hyperemia was noted, along with some

hepatic congestion. Hemorrhage in the lamina propria and tunica muscularis of the small intestine was observed which extended into the serosa. Trichrome staining indicated that the collagen appeared to be broken down in the submucosa of the duodenum. Within the pulmonary tissues it was noted that infarcts occurred with tissue loss and accumulation of pyknotic nuclei. Hemorrhage was also observed in the liver, pancreas, and kidney along with some hepatic thrombosis. Doses below the LD_{50} resulted in less severe pulmonary hemorrhage in regard to total area involved; however when death did occur, it involved confluent pulmonary hemorrhage. In all cases, the in vivo responses were proportional to the enzyme dosage.

Subcutaneous route. Administration of 24 to 309 collagenase units (27 to 343 μg protein) subcutaneously did not kill any of the mice. However, solubility and dosage limitations made it impossible to administer higher doses by this route. In addition, none of the animals showed the terminal signs of a lethal response such as ataxia and loss of caudal mobility. Injection of purified collagenase (24 to 309 units) into the abdomen or the tail resulted in the rapid destruction of skin and subcutaneous tissue at the injection site. Hemorrhaging lesions developed and became black, ulcerating, and necrotic almost immediately when 200 to 300 units of enzyme were administered. These lesions were reminiscent of the "ecthema gangrenosum" lesions seen in infected patients. Hemorrhage and necrosis of the abdomen or tail was observed with eventual loss of the skin from the tail at the site of inoculation. Doses as low as 45 enzyme units (50 μg protein) could produce the hemorrhage and

necrosis while 9 enzyme units (10 μg protein) produced redness and induration within 72 hr. In addition, the time of appearance of skin lesions appeared to be dependent upon the enzyme dosage. Histologically, large lesions and destruction of all skin layers reaching into subcutaneous tissues and the abdominal musculature could be observed along with hemorrhage and edema prevailing in the deeper regions. The collagen ground substance was actively altered or destroyed as detected by trichrome staining. Since the subcutaneous route never elicited death or gross pathological changes of the internal organs, no histological studies were undertaken on any of the organs.

No lethality or dermonecrosis was obtained with heat-inactivated collagenase by any of the routes used with the active enzyme preparations. The protein dosage for each route was identical to that used with the active enzyme.

TABLE 1

Summary of LD_{50} values for collagenase administered by various routes after 72 hr

	Route	Protein Concentration µg	Enzyme Concentration units
1.	Intranasal	61.8	55
2.	Intraperitoneal	164.8	148
3.	Intravenous	318.0	288
4.	Subcutaneous*	--	--

*No lethality was observed from 27 to 343 µg protein.